Altered expression of 3-containing GABAA receptors in the neocortex of patients with focal epilepsy

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Abstract: Impaired transmission in GABAergic circuits is thought to contribute to the pathogenesis of epilepsy. Although it is well established that major reorganization of GABAA receptor subtypes occurs in the hippocampus of patients with medically refractory temporal lobe epilepsy (TLE), it is unclear whether this disorder is also associated with alterations in GABAA receptor subtypes in the neocortex. Here we have investigated immunohistochemically the subunit composition and neocortical distribution of three major GABAA receptor subtypes using antibodies specifically recognizing the subunits 1, 2, 3, 2/3 and 2. Cortical tissue was obtained at surgery from patients with TLE and hippocampal sclerosis (HS; n = 9), TLE associated with neocortical lesions (non-HS; n = 12) and frontal lobe epilepsy (FLE; n = 5), with post-mortem samples serving as controls (n = 4). A distinct laminar and neuronal expression pattern of the -subunit variants was found across the neocortical regions examined in the temporal and frontal lobes in both control and patient tissue samples. In the five patients with FLE, GABAA receptor subunit staining was unchanged as compared to controls. In patients with TLE we observed a marked decrease in 3-subunit staining in the superficial neocortical layers (I-III), but no change in the deep layers (V and VI) or in the expression pattern of the 1 and 2-subunits. Reduced expression in 3-containing GABAA receptors was detected in six out of nine patients of the HS group and four out of twelve patients of the non-HS group. Histopathological changes were present in eight out of the ten patients with decreased 3-subunit staining. The selective reduction in 3-containing GABAA receptors was confirmed using semiquantitative measurements of optical density (OD). The specific changes unique to 3-subunit expression in the superficial neocortical layers of patients with TLE suggest that this subtype is of particular significance in the reorganization of cortical GABAergic systems in focal epilepsy

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Altered expression of α3-containing $GABA_A$ receptors in the neocortex of patients with focal epilepsy

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Impaired transmission in GABAergic circuits is thought to contribute to the pathogenesis of epilepsy. Although it is well established that major reorganization of $GABA_A$ receptor subtypes occurs in the hippocampus of patients with medically refractory temporal lobe epilepsy (TLE), it is unclear whether this disorder is also associated with alterations in $GABA_A$ receptor subtypes in the neocortex. Here we have investigated immunohistochemically the subunit composition and neocortical distribution of three major $GABA_A$ receptor subtypes using antibodies specifically recognizing the subunits α1, α2, α3, β2/3 and γ2. Cortical tissue was obtained at surgery from patients with TLE and hippocampal sclerosis (HS; *n* = 9), TLE associated with neocortical lesions (non-HS; *n* = 12) and frontal lobe epilepsy (FLE; *n* = 5), with post-mortem samples serving as controls (*n* = 4). A distinct laminar and neuronal expression pattern of the α-subunit variants was found across the neocortical regions examined in the temporal and frontal lobes in both control and patient tissue samples. In the five patients with FLE, $GABA_A$ receptor subunit staining was unchanged as compared to controls. In patients with TLE we observed a marked decrease in α3-subunit staining in the superficial neocortical layers (I–III), but no change in the deep layers (V and VI) or in the expression pattern of the α1 and α2-subunits. Reduced expression in α3-containing $GABA_A$ receptors was detected in six out of nine patients of the HS group and four out of twelve patients of the non-HS group. Histopathological changes were present in eight out of the ten patients with decreased α3-subunit staining. The selective reduction in α3-containing $GABA_A$ receptors was confirmed using semiquantitative measurements of optical density (OD). The specific changes unique to α3-subunit expression in the superficial neocortical layers of patients with TLE suggest that this subtype is of particular significance in the reorganization of cortical GABAergic systems in focal epilepsy.

Keywords: cerebral cortex; GABA; human; seizures; temporal lobe epilepsy

Abbreviations: ECoG = electrocorticography; FLE = frontal lobe epilepsy; HS = hippocampal sclerosis; OD = optical density; TLE = temporal lobe epilepsy

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Introduction

A variety of evidence indicates that inhibitory brain circuits, which depend primarily on signalling through $GABA_A$ receptors, are functionally impaired in epilepsy. Thus, blocking $GABA_A$ receptors pharmacologically promotes the generation of seizures in animals and in humans, whereas drugs that enhance $GABA_A$ receptor function are effective in treating seizures. This straightforward notion becomes more complex, however, when taking into account the wide array

of structurally and functionally distinct $GABA_A$ receptor subtypes found in the central nervous system. Nineteen $GABA_A$ receptor subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ, ρ1–3) have been identified and cloned from the mammalian CNS (Simon *et al.*, 2004), which theoretically can assemble into a vast number of distinct pentameric receptors. In fact, only a few dozen of these potential combinations are found in the brain, most of which include at least one of each of the α, β and γ subunit class (Fritschy and Möhler, 1995; Pirker *et al.*, 2000). Furthermore, these distinct receptor subtypes are preferentially expressed in specific regions and neuronal populations and they exhibit different sensitivities to modulators including neurosteroids, benzodiazepines, ethanol and barbiturates (Sieghart and Sperk, 2002; Fritschy and Brünig, 2003).

Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy in adults, and, when associated with hippocampal sclerosis (HS), is the most refractory to pharmacotherapy (Wieser and ILAE Commission on Neurosurgery of Epilepsy, 2004). Although abundant human data are available regarding reorganization of GABAergic interneuron circuits in the neocortex and hippocampus of TLE patients (DeFelipe, 1999; Sperk *et al.*, 2004; Maglóczky and Freund, 2005), most studies aimed at characterizing the alterations of individual $GABA_A$ receptor subtypes in human TLE have focused on the hippocampus (Brooks-Kayal *et al.*, 1999; Loup *et al.*, 2000; Pirker *et al.*, 2003; Porter *et al.*, 2005). Previous immunohistochemical investigations of $GABA_A$ receptors in the neocortex of patients with refractory focal epilepsy were restricted to the α1-subunit (Wolf *et al.*, 1994, 1996*b*). Results from autoradiographic investigations in the neocortex of patients with pharmacoresistant epilepsy yielded divergent conclusions, reporting no changes, decreases or increases in $GABA_A$ receptors (Olsen *et al.*, 1992; Burdette *et al.*, 1995; Nagy *et al.*, 1999; Zilles *et al.*, 1999; Sata *et al.*, 2002). *In vivo* imaging studies using ^{11}C-flumazenil, an antagonist at the benzodiazepine–$GABA_A$ receptor complex, have shown both focal increases and decreases of flumazenil binding in the neocortex of patients with partial epilepsy (Theodore, 2002; Koepp and Woermann, 2005). Although flumazenil-PET studies are indispensable for tracking changes in living subjects, the resulting images provide low spatial resolution of $GABA_A$ receptor distribution and flumazenil fails to distinguish between the different $GABA_A$ receptor subtypes. In the present study, we have used an immunohistochemical approach to investigate alterations in $GABA_A$ receptor subtype organization in neocortex removed at surgery from patients with medically intractable focal epilepsy. Tissue was processed following a protocol based on microwave irradiation to visualize the major $GABA_A$ receptor subunits α1, α2, α3, β2/3 and γ2 with subunit-specific antisera (Loup *et al.*, 1998). Other potentially relevant subunits could not be studied as the corresponding antisera effective in human brain tissue are not yet available.

Material and methods

Patient selection, intraoperative electrocorticography

Twenty-six patients undergoing surgery for medically intractable focal epilepsy were included in this study. Brain tissue was obtained from the neurosurgical units of the University Hospitals of Zurich, Geneva and Strasbourg. All procedures were performed with the informed consent of the patients or legal next of kin and were approved by the ethics committees of the respective institutions according to the Declaration of Helsinki. Presurgical assessment comprised high-resolution MRI, PET with ^{18}fluoro-2-deoxyglucose and/or ictal and interictal single photon emission computed tomography for all cases, functional MRI in six cases, and magnetic resonance spectroscopy (MRS) in five cases. All patients underwent scalp EEG and, where indicated, invasive or semi-invasive EEG recordings were obtained (Zumsteg and Wieser, 2000). Based on the histopathological findings in conjunction with neuroimaging, EEG and clinical data, patients were categorized into those with frontal lobe epilepsy (FLE) (n = 5, mean age at surgery ± SD: 22.6 ± 12.3 years, range 5–34 years) and those with TLE. The latter group was further subdivided into those with HS (n = 9, mean age at surgery ± SD: 39.6 ± 14.4 years, range 12–56 years) and those with neocortical lesions (non-HS; n = 12, mean age at surgery ± SD: 28.3 ± 12.2 years, range 9–49 years). Patients with focal cortical dysplasia (FCD) alone were specifically excluded. Relevant clinical data are summarized in Table 1.

Intraoperative electrocorticography (ECoG) was performed in 18 of 26 patients with grids of 4 × 8 electrodes and/or with strips of four electrodes (Pt/Ir) embedded in Silastic sheets (Ad-Tech, Racine, WI). In FLE patients, electrodes were placed over the exposed prefrontal and polar cortex and positioning of the grid and strip electrodes was modified to record from neighbouring areas where indicated. In TLE patients, the 4 × 8 grid was placed over the anterior and middle temporal lateral and inferior cortex and the strip electrode was positioned on the intraventricular hippocampus allowing for simultaneous recording from lateral and inferior temporal neocortex and from hippocampus. Centre-to-centre inter-electrode distance was 1 cm, the diameter of each electrode was 1.2 mm. Recordings were sampled at 400 Hz, with a bandwidth of 1–70 Hz over a minimum period of 25 min, using a 32-channel Nihon-Kohden EEG system. Spiking areas were defined where electrodes showed spikes and/or sharp waves with a mean frequency greater than 1 spike/min (+, 1–10; ++, 11–20; +++, > 20 spikes/min). Non-spiking areas were defined where electrodes showed no epileptiform graphoelements. Degree of spiking (+, ++, +++) was taken into account, as well as propagation if present. ECoG was carried out before resection and repeated during the surgical procedure as necessary. After surgical ablation, ECoG was performed again and showed either spike-free activity or residual spiking at the resection border. Residual spiking was anatomically localized and its degree was rated (+, ++, +++).

All tissue blocks were from resections performed for strictly therapeutic purposes. The surgical procedures included corticectomy or lesionectomy in the frontal or temporal lobe or anterior temporal lobectomy. Sixteen patients underwent amygdalo-hippocampectomy and HS was confirmed histopathologically in nine of these cases. The neocortical tissue samples originated from the anterolateral temporal neocortex and the frontopolar area, the frontal lateral region, and the orbital part of the inferior gyrus of the frontal lobe. In the patients with a circumscribed pathology, neocortical samples were collected within and adjacent to the

Table 1 Summary of clinical data and experimental results

Patient/ sex/age (years)	Location/ side	Age at onset	Duration of epilepsy	Seizure frequency at surgery	Seizure type	Prior exposure to barbiturates or BZs	Medication at time of surgery	ECoG neocortex	Histopathology	Postsurgical follow-up (months)	Engel outcome	α3-subunit changes in neocortex
1/F/48	T, R/L	—	—	—	—	—	—	Not done	Control	—	—	No
2/M/60	T/F, R/L	—	—	—	—	—	—	Not done	Control	—	—	No
3/M/69	T, L	—	—	—	—	—	—	Not done	Control	—	—	No
4/M/73	T, L	—	—	—	—	—	—	Not done	Control	—	—	No
5/M/5	F, R	1	4	20–30/night	CPS	—	LTG PHT VGB	+++	Mild subpial gliosis	71	IV	No
6/F/15	F, R	7	8	1–5/night	CPS SGS	PB CLB	BBC MSM CLB	+++	Venous angiitis	92	IV	No
7/M/27	F, R	23	4	3/month	CPS SGS	CLB	LTG VPA	++	Astrocytoma	86	I	No
8/M/34	F, R	12	22	20/month	CPS SGS	PRM CLB	LTG VPA OXC	++	Glial scar	85	III	No
9/M/32	F/T, L	3	29	4–10/month	CPS SGS	PB CLB	CBZ CZP	+++*	Glial scar	71	II	No
10/F/56	T, R	12	44	10/month	SPS CPS	PB	OXC CZP LEV	++	HS	56	I	No
11/F/49	T, L	18	31	15/month	CPS SGS	CLB	CBZ VGB	Not done	HS	62	I	No
12/M/56	T, L	15	41	6/month	CPS SGS	PRM	LTG CZP PRM	Non-spiking	HS	51	I	No
13/F/12	T, R	1	11	4/month	SPS CPS SGS	PB	STM	++ / +	HS	64	I	No/yes
14/F/35	T, R	8	27	7/month	CPS SGS	—	CBZ	+++	HS, mild MCD II	8	I	Yes
15/M/38	T, R	1	37	8/month	SPS CPS SGS	CLB	CBZ LTG	+++	HS, mild MCD II	56	I	Yes
16/M/25	T, R	15	10	5/month	CPS SGS	—	OXC LTG	+++	HS	40	I	Yes
17/F/40	T, R	5	35	2/month	CPS SGS	CLB	CBZ	Not done	HS	60	I	Yes
18/M/45	T, L	5	40	4/month	CPS SGS	CZP	CBZ CLB	Not done	HS	56	I	Yes
19/M/16	T, R	9	7	4/month	SPS CPS SGS	PB	OXC VPA	+++	Ependymoma	84	I	No
20/M/15	T, R	6	9	3/month	CPS	—	CBZ	++	Oligodendroglioma	42	I	No
21/M/49	T, L	32	17	8/month	CPS SGS	—	CBZ	Not done	Cavernoma	39	II	No
22/F/31	T, L	29	2	5/month	CPS	CLB	CBZ VPA	+	Cavernoma	43	I	No
23/M/43	T, L	11	32	4/month	SPS CPS	—	CBZ PHT	++	DNT	12	I	No
24/F/9	T, R	8	1	4/month	CPS	—	CBZ	+++	DNT	56	I	No
25/M/32	T, R	15	17	5/month	SPS CPS SGS	—	CBZ	++	Glial scar	64	I	No
26/M/27	T, R	18	9	5/month	CPS	—	CBZ	+++	Oligodendroglioma	81	I	Yes
27/M/22	T, R	15	7	8/month	CPS SGS	—	CBZ TGB	Not done	DNT, FCD IB	48	II	Yes
28/M/40	T, L	24	16	4–12/month	CPS SGS	PB	VPA TGB	++	Ganglioglioma, FCD IB	65	III	Yes
29/M/34	T, R	30	4	2/month	CPS	—	CBZ	Not done	Cavernoma	49	I	Yes
30/F/20	T, L	18	2	4/month	CPS	—	CBZ	Not done	DNT	48	I	Not done

BBC = barbexaclone; BZs = benzodiazepines; CBZ = carbamazepine; CLB = clobazam; CPS = complex partial seizures; CZP = clonazepam; DNT = dysembryoplastic neuroepithelial tumour; F = frontal; FCD = focal cortical dysplasia; HS = hippocampal sclerosis; LEV = levetiracetam; LTG = lamotrigine; mild MCD = mild malformation of cortical development; MSM = mesuximide; OXC = oxcarbazepine; PB = phenobarbital; PHT = phenytoin; PRM = primidone; SGS = secondary generalized seizures; SPS = simple partial seizures; T = temporal; TGB = tiagabine; TPM = topiramate; VGB = vigabatrin; VPA = valproic acid. Classification after Palmini and colleagues for mild MCDs and FCDs (Palmini *et al*., 2004).
*Preoperative intracranial recordings instead of ECoG.

lesion. Finally, control neocortical tissue from four subjects (mean age ± SD: 62.5 ± 11.1 years, range 48–73 years) with no known history of neurological or psychiatric disorders was collected at autopsy (mean post-mortem interval ± SD: 11.2 ± 3.4 h). Results on the hippocampi of these four subjects were reported in a previous study (Loup *et al.*, 2000). The left, and, in two cases, also the right hemispheres were cut into coronal slabs of 1–1.5 cm thickness. One to three tissue blocks per subject were dissected from parts of the anterolateral temporal neocortex, which corresponded to the areas removed in those patients undergoing surgery for epilepsy. In one case, three blocks of control tissue were also dissected from the frontal lobe.

Tissue preparation

Immediately upon resection in the operating room or after dissection at autopsy, tissue blocks were rinsed in phosphate-buffered saline (PBS) at pH 7.4. They were then immersion-fixed for 6–8 h at 4°C under constant agitation in a mixture of 4% freshly dissolved paraformaldehyde and 15% saturated picric acid in 0.15 M phosphate buffer at pH 7.4 (Somogyi and Takagi, 1982) or in 4% paraformaldehyde alone. Following fixation, tissue blocks were pre-treated using a modified antigen-retrieval method based on microwave irradiation as described previously (Loup *et al.*, 1998). Tissue blocks were cryoprotected in 10, 20 and 30% sucrose in PBS over a period of 3–4 days, frozen at −28°C in isopentane, and stored at −80°C. Series of 40 μm thick sections were subsequently cut in a cryostat and collected in ice-cold PBS. These were either processed for immunohistochemistry (see below) or transferred to antifreeze solution and stored at −20°C until use. This procedure allowed up to 12 different specimens to be processed in parallel. Staining for each of the five $GABA_A$ receptor subunits was always carried out on five consecutive sections with an interseries space of 720–800 μm. For histopathological examination, two additional adjacent series of sections were stained for Nissl with cresyl violet and with antibodies against the neuron-specific nuclear protein NeuN (Wolf *et al.*, 1996*a*). In selected cases, we also used antibodies against glial fibrillary acid protein (GFAP) and an antiserum that recognizes specifically a non-phosphorylated epitope of neurofilament protein (SMI-32) and labels a subpopulation of pyramidal cells (Campbell and Morrison, 1989).

Immunohistochemistry

The following subunit-specific antibodies were used: mouse monoclonal antibodies bd-24 and bd-17 recognizing the human $GABA_A$ receptor α1-subunit and both the β2 and β3-subunits, respectively (Schoch *et al.*, 1985; Ewert *et al.*, 1990), and polyclonal guinea-pig antisera recognizing the α2, α3 and γ2-subunits. The specificity of these antibodies has been extensively documented (Fritschy and Möhler, 1995; Loup *et al.*, 1998; Waldvogel *et al.*, 1999). The dilutions of the subunit-specific antibodies were: α1-subunit (monoclonal antibody bd-24), 0.14 μg/ml; α2-subunit (affinity-purified), 1.3 μg/ml; α3-subunit (crude serum), 1:3000; β2/3-subunit (monoclonal antibody bd-17), 3.8 μg/ml; and γ2-subunit (crude serum), 1:1500. Further, antibodies used were NeuN 1:1000 (MAB377; Chemicon, Temecula, CA), GFAP 1:100 000 (MAB360, Chemicon, Temecula, CA), and SMI-32 1:5000 (Sternberger Monoclonals Inc.; Covance Research Products, Berkeley, CA). Series of free-floating sections were pre-incubated in 1.5% H_2O_2 in PBS for 10 min at room temperature to block endogenous peroxidase activity. They were then washed three times for 10 min in PBS and processed for immunoperoxidase staining (Hsu *et al.*, 1981) as described previously (Loup *et al.*, 1998, 2000).

Data analysis

Sections were analysed with a Zeiss Axioskop 2 (Jena, Germany) equipped for bright-field microscopy. For display, images were digitized using a high-resolution camera (AxioCam; Zeiss, Jena, Germany) with Zeiss camera software (AxioVision version 4.4). Images were modified for contrast only and comparison images were adjusted uniformly (Adobe Photoshop version 7.0; Adobe Systems Incorporated, San José, CA). No other manipulation of images was performed. Illustrations were composed in Adobe Illustrator (version 10.0; Adobe Systems Incorporated, San José, CA).

Densitometric measurements

The intensity of labelling for the $GABA_A$ receptor subunits α1, α2, and α3 was measured by densitometry in sections from controls (n = 4), FLE (n = 4), HS (n = 8), and non-HS cases (n = 10) as described previously (Loup *et al.*, 2000). Optical density (OD) measurements were recorded in the superficial (layers II and III) and deep layers (layers V and VI) of the neocortex. The average OD per section was calculated from measurements in four rectangles with an area of 400 × 200 μm each in the superficial as well as in the deep layers and all measurements were repeated twice in sets of adjacent sections for each patient. In four patients, insufficient tissue was available to perform a complete quantitative analysis of the subunits.

Statistical analysis

Densitometric measurements were analysed for statistical significance using the Kruskal–Wallis test [non-parametric analysis of variance (ANOVA); GraphPad Prism, GraphPad Software, San Diego, CA]. Data were further compared between individual groups (at $P < 0.05$) with a multiple comparisons test. In the cases where more than one block was available, values were first subjected to statistical analysis to ensure that interblock variations were not significant.

Results

The distribution of the $GABA_A$ receptor subunits α1, α2, α3, β2/3 and γ2 was analysed in neocortical specimens from three different groups of patients with medically intractable focal epilepsy. Tissue obtained at autopsy from patients with no evidence of neurological disease was used for controls as previous data indicated that staining patterns for $GABA_A$ receptor subunits in autopsy and surgical samples are comparable (Loup *et al.*, 2000).

Patient histories and histopathological evaluation

Table 1 provides a summary of the relevant clinical data for each patient. The duration of epilepsy ranged from 4 to 29 years in the FLE group, from 10 to 44 years in the HS group and from 1 to 32 years in the non-HS group. Mean epilepsy duration was 13.4 years in the FLE group, 30.7 years in the

HS group and 10.6 years in the non-HS group. The mean age of onset was 9.2 years in the FLE group, 8.9 years in the HS group and 17.6 years in the non-HS group. In the HS group, but not in the FLE or non-HS groups, an initial precipitating event was documented as described previously (French *et al.*, 1993; Mathern *et al.*, 1995), including febrile convulsions ($n = 4$), infantile meningitis/encephalitis ($n = 2$) and neonatal anoxia/ischaemia ($n = 1$). The overall mean postsurgical follow-up period was 57.3 months. Seizure-free status (class I; Engel, 1987) was achieved for all patients of the HS group, 9 out of 12 patients in the non-HS group and 1 patient of the FLE group.

With respect to the patients with FLE, histopathological examination revealed discrete focal Chaslin's subpial gliosis ($n = 1$), leptomeningeal venous angiitis with no abnormalities in the brain parenchyma ($n = 1$), a low grade tumour ($n = 1$), and a glial scar ($n = 2$). In the patients with histopathologically confirmed HS, examination of the anterolateral temporal neocortex showed mild Chaslin's subpial gliosis ($n = 3$), gliosis ($n = 4$) and/or white matter changes. Thus, in two cases, small aggregates of heterotopic neurons were detected in the subcortical white matter. According to Palmini *et al.* (2004), such abnormalities are classified as type II mild malformations of cortical development (mild MCD II). In contrast, in neocortical grey matter, staining for Nissl, NeuN or SMI-32 revealed a normal cytoarchitecture with no apparent neuronal cell loss (*see* Fig. 4A and D). The non-HS group consisted of patients with vascular cavernous malformations ($n = 3$), low grade tumours ($n = 8$) and a glial scar secondary to cranial trauma ($n = 1$). All lesions were located in the anterolateral temporal neocortex. In 4 of the 12 patients with non-HS we also found histopathological changes in tissue adjacent to the lesion. In particular, dyslamination and large neurons were observed in two cases, one with a dysembryoplastic neuroepithelial tumour (DNT) and the other with a ganglioglioma. According to Palmini *et al.* (2004), the abnormalities described above are consistent with type IB (FCD IB). Neocortical tissue from autopsy controls displayed a normal cytoarchitecture.

$GABA_A$ receptor subtypes in control grey matter

We first examined the distribution of the $GABA_A$ receptor subunits α1, α2, α3, β2/3 and γ2 in temporal neocortex at low power magnification (Fig. 1). Adjacent sections were stained for the neuronal marker NeuN to identify laminar borders. All subunit antibodies revealed specific patterns of immunoreactivity. In normal grey matter, the laminar pattern for the subunits α1, α2 and α3 was distinct, whereas the laminar pattern was similar for the subunits β2/3 and γ2 (Fig. 1). A comparable organization was also described in rodent brain where the α-subunit variants represent largely distinct subtypes with a specific pharmacological profile while the β2/3 and γ2-subunits are common to most

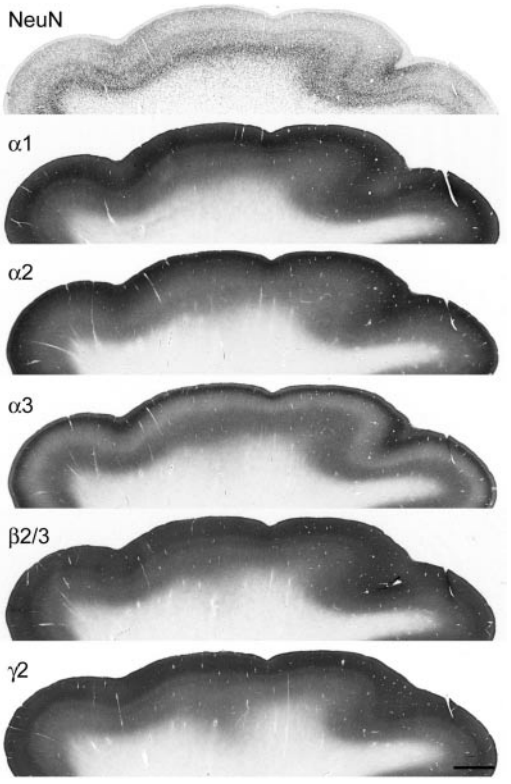

Fig. 1 Regional distribution of the major $GABA_A$ receptor subunits in temporal neocortical tissue obtained at autopsy from a control. Adjacent sections were stained for the subunits α1, α2, α3, β2/3 and γ2, and for NeuN. The NeuN staining illustrates the laminar distribution of neuronal cell somata. Each of the α-subunit variants exhibits a distinct laminar distribution whereas labelling for the β2/3 and γ2-subunits is largely similar. Note that the α1-subunit has a nearly identical distribution pattern to that of the β2/3 and γ2-subunits. Scale bar: 2 mm.

$GABA_A$ receptor subtypes (Fritschy and Möhler, 1995). Furthermore, the distribution pattern for the α1-subunit was nearly identical to that of the β2/3 and γ2-subunits, indicating that the α1-subunit is the most abundant of the α-subunit variants in the human neocortex. Unless otherwise mentioned, the ubiquitously present subunits β2/3 and γ2, although analysed, will not be further described.

Figure 2 shows at higher magnification the distinct and specific pattern of laminar distribution in neocortex of each of the α-subunit variants tested. Staining for the α1-subunit was particularly intense in the lower part of layer III and in layer IV (Fig. 2B). A weaker labelled band corresponding to the upper/mid layer IV in adjacent NeuN and SMI-32-stained sections was often observed (Fig. 2A and B) and was best seen at low power magnification (Fig. 1). For the α2-subunit, labelling was pronounced in the superficial layers and weak in the deep layers (Figs. 1 and 2C). A transition in labelling intensity generally was apparent in layer IV. Immunoreactivity for the α3-subunit was most intense in layer II and the upper part of layer III, gradually decreasing

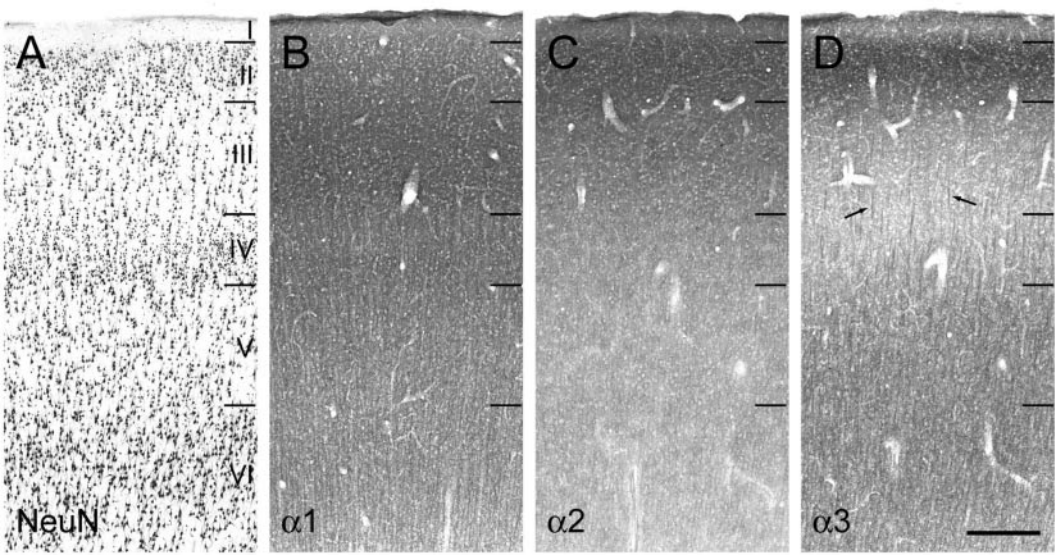

Fig. 2 Differential laminar distribution of $GABA_A$ receptor subunit immunoreactivity in control human temporal cortex. (**A**) NeuN-stained section. The characteristic six-layered structure of neocortex (I–VI) was used to define the laminar borders in adjacent immunostained sections. (**B**) α1-Subunit immunoreactivity is present throughout the six layers, but most intense in the lower part of layer III and IV. A lighter band of staining beneath layer III corresponds to the upper/mid part of layer IV. Note the staining of the neuropil, in which individual neurons cannot be distinguished at this magnification. (**C**) α2-subunit immunoreactivity is most abundant in the superficial layers (I–III), decreases progressively (III and IV) and becomes weakest in layers V and VI. (**D**) α3-Subunit immunoreactivity is most intense in layer II and the upper part of layer III, light in layer IV and moderate in layers V and VI. Labelled apical dendrites originating from layer V and VI pyramidal cells and extending towards the superficial layers (arrows) are observed even at this low magnification. Scale bar: 400 μm.

toward the lower part of layer III. Layer IV was only lightly labelled (Figs. 1 and 2D). The deep layers were moderately labelled. Even at this power of magnification, apical dendrites originating from layers V and VI could be seen to extend toward the pial surface (Fig. 2D). For each subunit, similar patterns of staining were observed in temporal and frontal neocortical tissue of all autopsy cases (data not shown).

At the cellular level, staining for the α2-subunit was present in the neuropil of each layer with the neuronal cell bodies appearing lightly labelled against the background (Fig. 3A). α3-Subunit immunoreactivity was also observed in the neuropil, but additionally in the soma and dendrites of individual neurons, including in pyramidal cells predominantly situated in the lower part of layer III (Fig. 3B). In layers V and VI, a subpopulation of neurons was labelled that displayed the typical morphology of pyramidal cells with a few basal dendrites and long apical dendrites coursing toward the superficial layers (Fig. 3C). Figure 3D shows a high magnification image of a layer V pyramidal cell immunoreactive for the α3-subunit. For both the α2 and α3-subunits, intense immunoreactivity was also detected on the axon initial segment of pyramidal cells, as shown previously (Loup *et al.*, 1998). Staining for the α1-subunit, and less prominently for the β2/3 and γ2-subunits, was not only seen in the neuropil, but also in numerous non-pyramidal cells. In particular layer II and the upper part of layer III displayed a high density of intensely stained interneurons with somata of small size and several, often radially oriented, dendrites (Fig. 3E).

$GABA_A$ receptor subtypes in the grey matter of patients with focal epilepsy

Neocortical tissue from patients with FLE, HS or non-HS was analysed for changes in $GABA_A$ receptor subunit immunoreactivity. In the patients with a circumscribed lesion in the neocortex (*see* Table 1), the tissue samples used for study were from the periphery of the lesion. Two major observations were made. First, in all three groups the staining pattern for the subunits α1 and α2, as well as β2/3 and γ2, was largely similar to that of controls in terms of laminar distribution and intensity (Fig. 4B, C and E). Secondly, a subset of patients (10/26) was found to exhibit markedly decreased α3-subunit staining in the superficial layers, whereas the deep layers appeared unchanged and thus provided an internal control within each section for each case (Figs. 4F and 5). In the patients with reduced α3-subunit staining, no apparent neuronal cell loss was observed in adjacent sections stained for SMI-32 or NeuN (Fig. 4A and D). The data for the α3-subunit in the superficial neocortical layers for each patient are summarized in Table 1.

In the group of patients with FLE (n = 5), no changes in α3-subunit immunoreactivity were observed as compared to controls. In the HS group (n = 9), α3-subunit

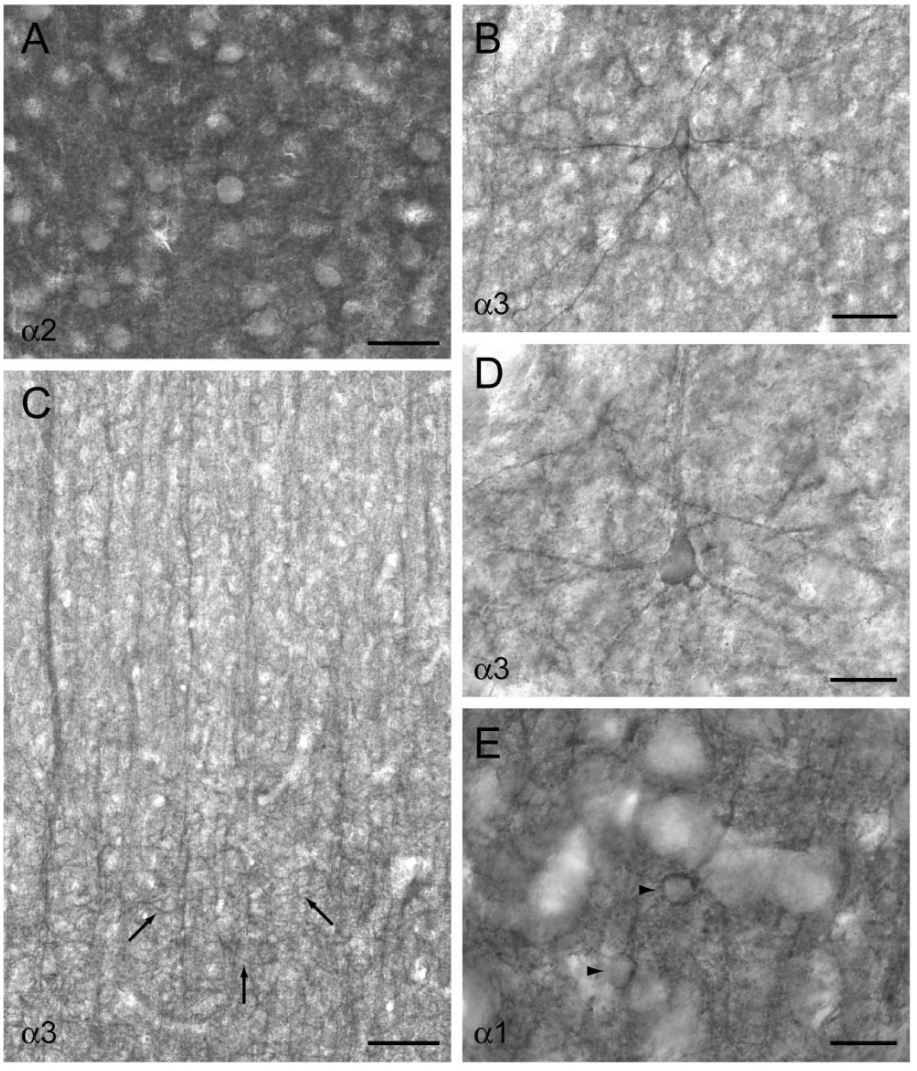

Fig. 3 Differential cellular distribution of $GABA_A$ receptor subunit immunoreactivity in control human temporal neocortex. (**A**) α2-Subunit staining in the neuropil. The lightly labelled round structures represent pyramidal cell somata located in layer II. (**B**–**D**) α3-Subunit immunoreactivity. In **B**, a pyramidal cell in the lower part of layer III exhibits strong staining of the soma membrane and long dendrites whereas the surrounding neuropil is weakly stained. (**C**) Several layer V pyramidal cells with basal (arrows) and long apical dendrites that extend throughout layer IV toward the pial surface. (**D**) High magnification of a layer V pyramidal cell with one apical and several basal dendrites against the diffusely stained background. (**E**) α1-Subunit immunoreactivity. Prominent labelling against the diffusely stained neuropil of two small interneurons (arrowheads), one of which displays radial dendrites. The large and lightly labelled structures represent pyramidal cell somata. Scale bar: (**A** and **B**) 50 μm; (**C**) 100 μm; (**D** and **E**) 20 μm.

immunoreactivity was unchanged in three and decreased in five cases. In the remaining case, regions of unchanged and decreased α3-subunit staining were seen in the superficial layers in two different samples while no alterations were observed in α3-subunit staining in the deep layers. In the non-HS group ($n = 12$), α3-subunit labelling was unchanged in seven cases, decreased in the superficial layers in four cases, and could not be determined in one case. Fig. 5 shows low power images of the laminar distribution pattern of the α3-subunit from six different cases (five HS, one non-HS), three of which had no changes and three of which had decreased α3-subunit staining in the superficial neocortical layers.

Semiquantitative densitometric analysis was performed to assess differences in staining intensity between the control, FLE, HS and non-HS groups for the subunits α1, α2 and α3 in the superficial and the deep layers of temporal or frontal neocortical tissue (Fig. 6). The following observations were made: (i) no significant differences in OD were found for the α1 and α2-subunits in the superficial or the deep layers between the different groups of patients (Fig. 6A–D). (ii) Differences in OD for the α3-subunit were observed in the superficial layers of a subset of patients in both the HS and the non-HS groups (Fig. 6E). When compared to the FLE group, α3-subunit OD was significantly decreased in both the HS and the non-HS groups ($P < 0.01$ and $P < 0.05$,

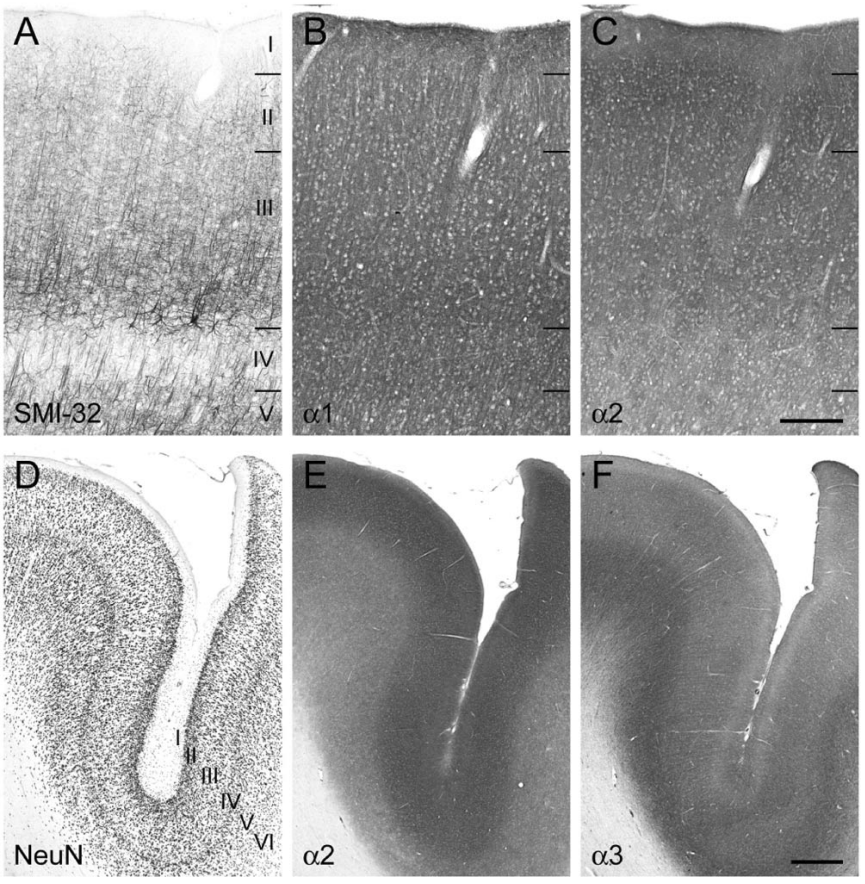

Fig. 4 Subunit-specific changes in $GABA_A$ receptor immunoreactivity in the temporal neocortex from two TLE patients. (**A**–**C**) Adjacent sections stained for SMI-32 (**A**), the α1-subunit (**B**) and the α2-subunit (**C**). The distribution pattern of these subunits is unchanged as compared to controls (*see* Figs. 1 and 2). (**D**–**F**) Adjacent sections stained for NeuN (**D**), the α2-subunit (**E**) and the α3-subunit (**F**). The cytoarchitecture is conserved with no apparent neuronal cell loss. Note that layer-specific changes occur only for the α3-subunit, which is decreased in the superficial layers and unchanged in the deep layers. Scale bar: (**A**–**C**) 300 μm; (**D**–**F**) 1 mm.

respectively). Comparison within the HS group and within the non-HS group showed that α3-subunit OD was significantly decreased in a subset of patients ($P < 0.0001$ and $P < 0.0001$, respectively). (iii) No significant difference in OD was found for the α3-subunit in the deep layers for all patients (Fig. 6F).

At the cellular level, sections stained for the subunits α1 and α2 revealed similar patterns in all specimens when compared to controls (data not shown). For the α3-subunit, Fig. 7A shows a section from a case with an unchanged pattern of staining similar to what was seen in control tissue. In the HS and non-HS patients with changes in α3-subunit immunoreactivity in the superficial neocortical layers, a striking decrease in staining was present throughout layers I, II and III (Fig. 7B–D). Moreover, in layer II only, we observed pyramidal cells immunopositive for the α3-subunit, which had apical dendrites extending into layer I (Fig. 7C and D). These neurons possessed numerous long dendrites, which at times formed an intricate predominantly horizontal network with neighbouring layer II neurons (Fig. 7C). Some cells had a prominently labelled soma (Fig. 7D). Changes at the cellular level as depicted in Fig. 7 were seen to a variable degree in the temporal neocortex of all patients with reduced α3-subunit staining.

Intraoperative ECoG, changes in α3-containing $GABA_A$ receptors and histopathology

ECoG was performed during surgery in 18 of 26 patients and results were analysed in a semiquantitative manner as described in Material and methods. As can be seen in Table 1, no consistent correlation was found between the degree of spiking activity recorded before resection and the changes in α3-subunit staining or the histopathology. In particular, areas with high spiking activity (+++) exhibited either unchanged or decreased α3-subunit expression. The post-resection ECoG recordings showed that no or only little residual spiking was present at the border of the resection except in one FLE patient with rhythmic discharges in the contralateral frontal neocortex.

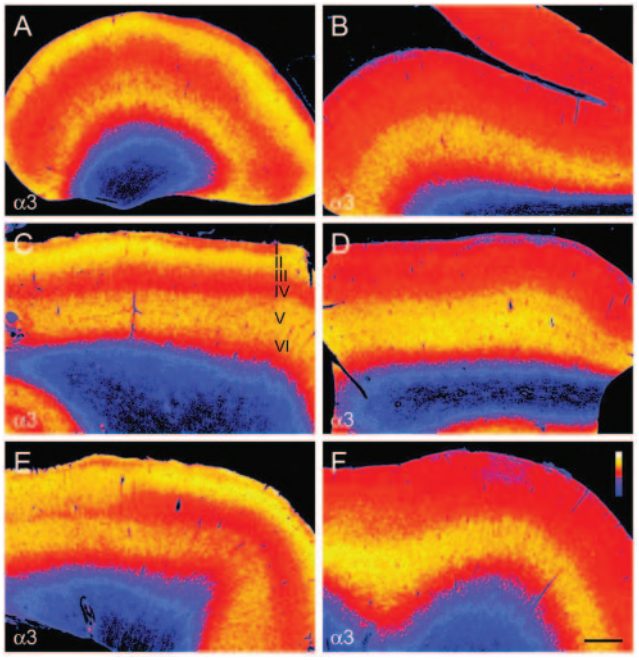

Fig. 5 $GABA_A$ receptor α3-subunit immunoreactivity in the temporal neocortex from six TLE patients. The colour-coding indicates OD of staining using a normalized scale with the strongest signal in white and no signal (background) in dark blue. (**A**, **C** and **E**) α3-subunit immunoreactivity in three patients with HS showing no change in the laminar distribution of this subunit. (**B**, **D** and **F**) In two other patients with HS (**B** and **D**) and one with non-HS (**F**), staining for the α3-subunit is decreased in the superficial layers and unchanged in the deep layers. Scale bar: 1 mm.

Discussion

Our first principal finding is that in the normal human neocortex the expression of five of the major $GABA_A$ receptor subunits (α1, α2, α3, β2/3 and γ2) displayed remarkable laminar and neuronal specificity. Moreover, the regional distribution of the $GABA_A$ receptor subunits was similar across all the neocortical regions examined in the temporal and frontal lobes. Second, in a subset of patients with TLE we observed a decrease in α3-subunit staining in the superficial neocortical layers, usually accompanied by histopathological changes. In contrast, the distribution and the intensity of labelling of the subunits α1, α2, β2/3 and γ2 were unchanged in patients with focal epilepsy.

Methodological considerations

The feasibility of this study depended on the utilization of high affinity antibodies and the quality and processing of the tissue. We verified the specificity of the subunit-specific antisera in human brain tissue in previous studies with competition experiments, by replacing primary antibodies with non-immune serum and in western blots (Loup *et al.*, 1998; Waldvogel *et al.*, 1999). Moreover, to achieve high specificity of staining with a low background, we used an antigen-retrieval microwave procedure adapted to human brain tissue (Loup *et al.*, 1998).

A number of potential pitfalls must be considered in the interpretation of our findings. (i) Variability, because tissue samples originated from different epilepsy centres. However, tissue processing was performed according to a common protocol (Loup *et al.*, 1998, 2000) and the specific reduction in α3-subunit staining was found in tissue samples from all three centres. (ii) A sampling problem, in that areas compared between different patients were in fact not homologous. This is rather unlikely as the location of the resected neocortical areas was carefully documented and the distribution of the subunits α1 and α2, which served as a reference, was unchanged across samples. (iii) Effects of confounding biographical/clinical data, in particular differences in pharmacotherapy or the occurrence of presurgical seizures (Bouvard *et al.*, 2005). Thorough review of the clinical data from each patient failed to identify major discrepancies among patients. Furthermore, it is unlikely that the changes in α3-subunit expression are secondary to drug treatment for the following reasons: a decrease in α3-subunit expression was observed in tissue both from patients taking GABAergic drugs and from patients not taking GABAergic drugs. In other words, there was no correlation. Also, at least four patients who were not treated with GABAergic drugs at the time of surgery and with no history of prior exposure nevertheless exhibited decreased α3-subunit staining. Moreover, in patients in whom α3-subunit expression was decreased in temporal neocortex, the expression in the entorhinal cortex was unchanged (F. Loup, unpublished data), whereas all five subunits studied including the α3-subunit were differentially altered in the hippocampus (Loup *et al.*, 2000). (iv) Staining artefacts. This possibility can be ruled out based on our uniform processing in parallel of the various tissue samples. In addition, immunohistochemical staining in the cases with decreased α3-subunit labeling was repeated several times and, finally, staining in the deep layers was unchanged, thus providing an intrasection control.

$GABA_A$ receptor subtype expression in normal grey matter

Among the three $GABA_A$ receptor subtypes identified in our study, the α1-subtype was most abundant, with highest staining in lower layer III and layer IV, followed by the α2 and the α3-subtypes. This is in line with the results of previous autoradiographic $GABA_A$ receptor binding studies (Young and Kuhar, 1979; Zezula *et al.*, 1988) reporting high densities of benzodiazepine receptor sites, especially in layers III and IV. Using *in situ* hybridization for six $GABA_A$ receptor subunit mRNAs (α1, α2, α5, β1, β2 and γ2), Akbarian *et al.* (1995) found three principal patterns of laminar expression in normal human prefrontal cortex, with the α1-subtype dominating. The laminar distribution pattern that they reported for the α1, α2, β2 and γ2-subunit mRNAs is largely

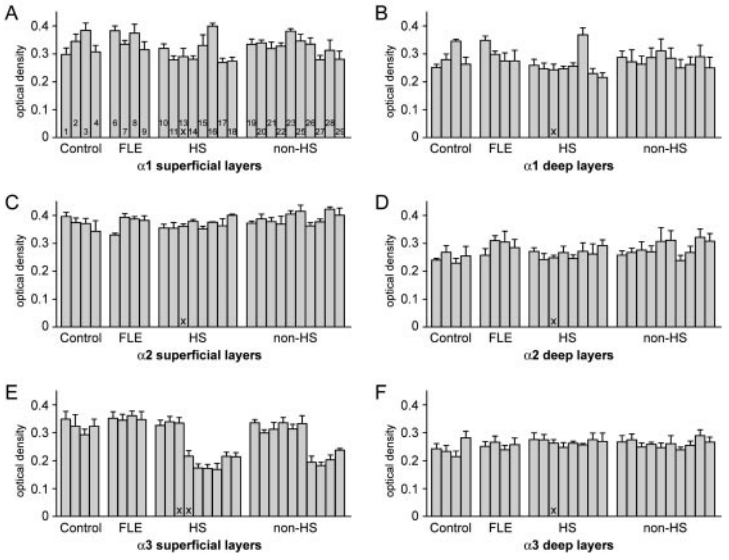

Fig. 6 Densitometric measurements (mean ± SD) in four controls, 4 FLE, 8 HS and 10 non-HS patients showing that a subset exhibits decreased α3-subunit staining in the superficial neocortical layers (13–18, 26–29). Sets of three immediately adjacent sections stained for the subunits α1 (**A** and **B**), α2 (**C** and **D**) and α3 (**E** and **F**) were used. OD measurements were made in layers II and III (**A**, **C** and **E**) and in layers V and VI (**B**, **D** and **F**) of the same section. The numbers 1–29 indicated in **A** refer to each of the patients listed in Table 1. One exception is number 13 (marked with an x). In tissue from this patient, densitometric measurements of α3-subunit staining in the superficial layers showed a lack of change in one sample and decreased staining in the other (**E**, xx). In contrast, all the other densitometric measurements in this patient were uniform between blocks as reflected by the low SDs (**A**–**D** and **F**).

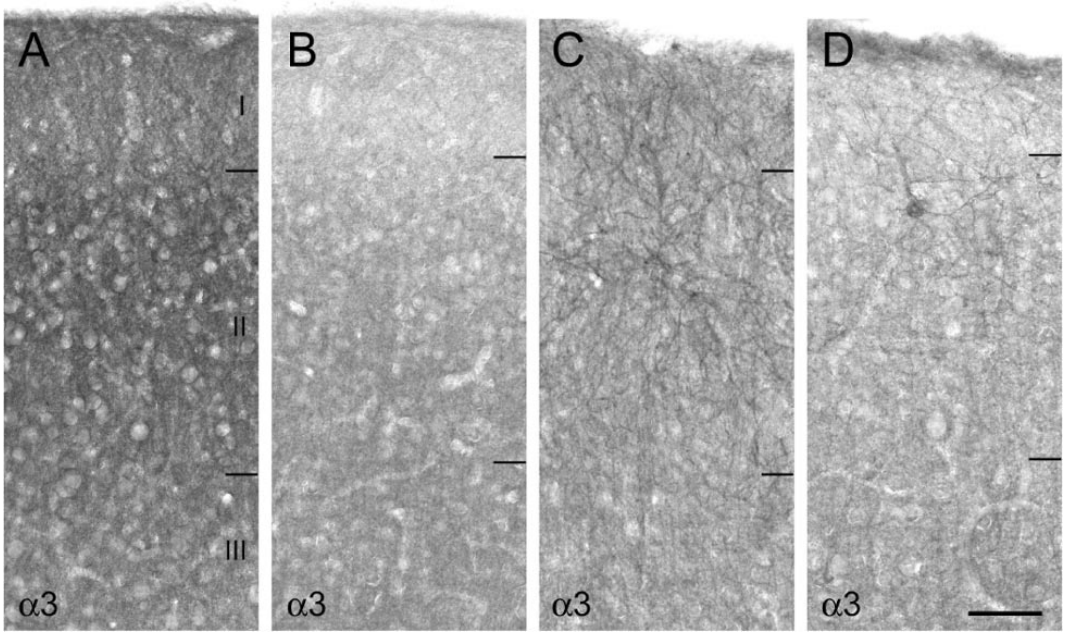

Fig. 7 Variability of α3-subunit immunoreactivity at the cellular level in the superficial neocortical layers in TLE specimens with decreased α3-subunit staining (**B**–**D**) versus a TLE specimen with unchanged labelling (**A**). (**A**) α3-Subunit immunoreactivity showing strong neuropil staining in layer II and weaker staining in the upper part of layer III. In (**B**), α3-subunit labelling is decreased throughout the superficial layers. (**C**) Section showing a layer II pyramidal cell with an extensive dendritic arborization restricted to layers I and II. (**D**) Section showing a large pyramidal cell also located in layer II revealed by α3-subunit immunoreactivity. Scale bar: 100 μm.

similar to that described in the present immunohistochemical study. Moreover, the $GABA_A$ receptor subunits α1, β2/3 and γ2 show a similar laminar distribution in the human visual cortex with immunoreactivity greatest in layer IV (Hendry *et al.*, 1994). Finally, electrophysiological data from a study in human temporal cortical neurons demonstrated a benzodiazepine sensitivity profile consistent with a preponderance of α1-subunit expression (Gibbs *et al.*, 1996).

To our knowledge, the present study provides the first description of α3-subunit immunoreactivity in the human neocortex, revealing a remarkably specific laminar distribution pattern. This subunit is especially interesting because, in contrast to the other subunits, its expression differs from that described in the rodent brain (Fritschy and Möhler, 1995; Pirker *et al.*, 2000). In rodent neocortex, α3-subunit expression is mainly located in the deep layers, while in the human neocortical frontal and temporal regions examined, α3-subunit staining was also pronounced in the superficial layers, especially in layer II. Our results further demonstrate a cell-specific distribution of the five major $GABA_A$ receptor subunits. The α1 together with the β2/3 and the γ2-subunits were localized not only in pyramidal cells but also in numerous small interneurons, especially in the superficial layers. Layer II and the upper part of layer III of the human temporal neocortex contain smaller and a higher density of GABA-positive somata than the other layers (Kisvárday *et al.*, 1990). In contrast, the α2 and α3-subunits were localized solely in pyramidal cells, most prominently at the axon initial segment (Loup *et al.*, 1998; Volk *et al.*, 2002).

Altered $GABA_A$ receptor subtype expression in the grey matter of patients with focal epilepsy

We found a selective reduction of the α3-subunit in the neocortex of a subset of TLE patients in the absence of a change of the α1, α2, β2/3 and γ2-subunits. Data from animal studies indicate, however, that functional α3-containing $GABA_A$ receptors generally co-assemble with β and γ2-subunits (Sieghart and Sperk, 2002; Fritschy and Brünig, 2003). The reason that we did not detect an associated decrease in β2/3 and γ2-subunit labelling probably relates to the ubiquitous distribution of these subunits. The β and γ2-subunits are included in the vast majority of $GABA_A$ receptors, most frequently in combination with the α1 or α2-subunit. Thus, a decrease in the fraction of β and γ2-subunits co-expressed with the α3-subunit, which in rodent brain constitutes only 10–20% of the total number of $GABA_A$ receptors (Sieghart and Sperk, 2002; Fritschy and Brünig, 2003), would result in a change too low to be detected by immunohistochemical methods.

Among all known subunits the α1 is the only one previously analysed immunohistochemically in the temporal neocortex of patients with pharmacoresistant focal epilepsy (Wolf *et al.*, 1994, 1996*b*). In their first study where the primary focus was the hippocampal formation, Wolf *et al.* (1994) used the temporal neocortex as a reference and reported no changes in α1-subunit $GABA_A$ receptor immunoreactivity, which is confirmed by our data. Autoradiographic analysis of binding at $GABA_A$ receptors or at the benzodiazepine–$GABA_A$ receptor complex in the temporal neocortex of patients with medically refractory focal epilepsy has provided conflicting findings. Thus Zilles *et al.* (1999) using ^{3}H-muscimol reported a downregulation of $GABA_A$ receptor density in four out of nine cases with non-HS and variable upregulation in the remaining cases. In TLE patients with HS, Olsen *et al.* (1992) found no significant difference in flumazenil binding between epileptic temporal neocortex and control tissue, whereas Burdette *et al.* (1995) reported a significant increase in flumazenil binding in layers V and VI of epileptic temporal neocortex. Taken together these studies show non-uniform changes in $GABA_A$ receptor binding in tissue from TLE patients. Similarly, imaging studies using ^{11}C-flumazenil in patients with refractory focal epilepsy due to diverse pathologies have reported not only focal decreases but also focal increases of flumazenil binding either in temporal or extratemporal neocortex (Theodore, 2002; Koepp and Woermann, 2005). Until subtype-specific ligands become available it will not be possible to determine how the changes in α3-subunit expression observed in the present work relate to the changes described in autoradiography and PET studies.

In our study, the non-HS group consisted of patients with a focal lesion located in the anterolateral temporal neocortex. When we examined the neocortex adjacent to the lesion, we observed that it was histologically normal in 8 of the 12 cases where we also did not find any changes in α3-subunit staining compared to controls. In the other four patients with decreased α3-subunit expression, we found histopathological changes including gliosis and type IB FCD. This type of mild MCD was present in one patient with a DNT and one patient with a ganglioglioma. Both types of tumours may be associated with surrounding dysplastic cortical regions as described previously (Prayson *et al.*, 1993; Daumas-Duport *et al.*, 1999; Palmini *et al.*, 2004). Wolf *et al.* (1996*b*) reported decreases as well as increases in α1-subunit immunoreactivity in the perilesional tissue in a subset of patients with neocortical lesions. We did not find alterations in α1-subunit staining in our study, but overall these results show that $GABA_A$ receptor changes can occur in the vicinity of focal lesions.

The HS group comprised nine patients, three with no change in α3-subunit labelling, five with decreased labelling and one with a decrease in one of two tissue samples. Mild Chaslin's subpial gliosis was detected in two of the patients with no change in α3-subunit labelling and in the patient where there was reduced staining in one of the two samples. In contrast, in four of the five cases with decreased α3-subunit staining, we observed gliosis and/or white matter changes, two of whom exhibited type II mild MCD (Palmini *et al.*, 2004). Thus, decreased α3-subunit expression in patients with HS or with non-HS was associated in 8 out of 10 patients with histopathological changes, in particular mild forms of MCD. We did not, however, observe neuronal loss in TLE specimens, in agreement with previous studies (Babb *et al.*, 1984; Bothwell *et al.*, 2001). These findings suggest that the reduction in α3-subunit staining is related primarily to a downregulation of this subunit in pyramidal cells rather than to a loss of neurons expressing this subunit.

Furthermore, decreased α3-subunit expression was not accompanied by a reduction in the α1 or α2-subunits, which would be expected were the changes a result of extensive neuronal loss.

We also studied five patients with FLE, where we did not find any changes in $GABA_A$ receptor subunit staining. Whether this lack of change reflects the small sample size, the type of pathology (absence of a circumscribed lesion except in one case) or other factors remains unclear. It is however interesting to note that the laminar distribution and intensity of $GABA_A$ receptor subunits was basically similar to that in the autopsy specimens and TLE samples.

In the patients with altered α3-subunit expression, the laminar pattern was remarkably stereotyped with a lack of change in deep layers and a marked decrease in staining in superficial layers. At the cellular level it is not possible to determine whether the more darkly stained, and therefore visible, pyramidal cells were the last cells to express the α3-subunit or whether these neurons expressed this subunit *de novo*. Nevertheless, the selective localization in layer II of intensely stained neurons with an extensive dendritic arborization confined to layers I and II suggests a reorganization of the α3-subtype in these layers. A recent study using complementary DNA microarrays and immunostaining reported a pattern of persistent gene activation in epileptic neocortex of patients with focal epilepsy mainly in layers II and III (Rakhade *et al.*, 2005). As the superficial layers are primarily involved in processing activity from other cortical areas (Jones, 1984) and electrophysiological studies have shown the critical role of layers II and III in the generation of synchronous population events in human epileptic neocortex (Köhling *et al.*, 1999), downregulation of α3-containing $GABA_A$ receptors in the superficial layers may contribute to decreased functional inhibition.

Other $GABA_A$ receptor subunits, for which antibodies effective in human tissue do not yet exist, might be upregulated and may thus compensate for a reduction in α3-subunit expression. However, a recent study using transcriptome profiling in human epileptic neocortex reported a prominent downregulation of the α5-subunit gene and other GABA system transcripts in both the presynaptic and the post-synaptic compartments in spiking samples (Arion *et al.*, 2006). Further, functional and morphological alterations in GABAergic circuits in the neocortex of TLE patients have been described previously (DeFelipe, 1999; Avoli *et al.*, 2005). In particular, the group of DeFelipe found patterns of decreased immunoreactivity for parvalbumin (PV) and glutamate decarboxylase in human epileptogenic neocortex (DeFelipe *et al.*, 1993, 1994; Marco *et al.*, 1996). The most important change was a focal loss of PV-positive chandelier cells (among other interneurons), which are thought to be the most powerful cortical inhibitory cells. Using quantitative electron microscopy, the same group found a loss of inhibitory synapses in the regions with marked decrease in staining for PV (Marco and DeFelipe, 1997). Interestingly, the changes in synaptic density in their study were most pronounced in the superficial layers, where we observed downregulation of α3-containing $GABA_A$ receptors. Taken together, these results suggest a prominent reorganization of the GABAergic circuitry, which could contribute to the genesis or the maintenance of seizure activity in human focal epilepsy.

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